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Enhanced production of prodigiosin, a potent compound from *Serratia marcescens* against Methicillin Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

The red pigment, prodigiosin derived from *Serratia marcescens* is a most convincing drug with immunosuppressive, antibacterial, antifungal and anti- proliferative properties. In the present work investigations were carried on to effectively isolate the prodigiosin producing strain from the soil sample, which is followed by the pigment production and characterization of its antimicrobial activity respectively. The maximum yield of prodigiosin was attained by *Serratia marcescens* when the medium was maintained at the pH 7 (585.54mg/L) and while the medium was incubated at 28°C (593.12mg/L). Along with this, as the medium was provided with 1.5% salt concentration significant amount of prodigiosin (585.81mg/L) was achieved. Similarly, as the prodigiosin producing medium was supplemented with the additional carbon source like sucrose 576.76 mg/L of pigment was produced. Among the oil supplemented medium, maximum prodigiosin productivity (536.04 mg/L) was achieved while coconut oil was provided. The pigment prodigiosin was found successful in inhibiting Methicillin – resistant *Staphylococcus aureus* at 150µl (8mm).

INTRODUCTION

Prodigiosin is a red pigment produced by the bacterium *Serratia marcescens* is an arising significant particle due to its enormous application. Prodigiosin is a direct tripyrrole red coloured bioactive secondary metabolite that commonly collects on the cell membrane and intracellular granules. These properties of pigmented compounds like anticancer, antifungal, algicidal, antimalarial are still to be examined. It has been demonstrated that pigmented strain of *Serratia marcescens* is less harmful than non-pigmented strain which would be affirmed by morphological, biochemical, and phylogenetic examinations. Thus, these non-pigmented strain would be utilized as negative control. Despite the fact that *S. marcescens* is a significant producer of prodigiosin, this pigment is also found to be produced by other microbes like *Streptomyces coelicolor*, *S. lividans*, *Hahella chejuensi*, *Psuedovibrio denitricans*, *Psuedoaltero monasrubra*, *P. denrificans*, *Vibro gazogenes*, *V.psychroerythreus*, *Serratia plymuthica*, and *Zooshikella rubidus* [1].

Serratia marcescens is a gram negative, motile, short rods and facultative anaerobe bacterium, which was considered an opportunistic pathogen found in 1819 by Bartolomeo Bizio in Padua, Italy [2]. These organisms show ubiquitous presence in the climate with an inclined damp condition and the *S. marcescens* grows readily at 37° c and pH 7. It is a facultative microorganism and accordingly the pigment is developed under both aerobic and anaerobic conditions. *Serratia marcescens* is highly notable for the red pigmentation it produces called prodigiosin. A few strains found to produce red pigment, though others do not produce the pigment. Numerous kinds of selective

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and differential media have been utilized to enhance the growth of *Serratia sp.* and the prodigiosin development.

The red color prodigiosin was successfully secluded from *S. marcescens* in the year of 1902 by Kraft [3]. The pigment was discovered to be 10% and was affirmed spectrophotometrically [4]. Prodigiosin are the very strong therapeutic compounds with immunosuppressive and anticancer properties. The action mechanism of the prodigiosin compound was suggested by Perez-Tomas *et al.* [5] and the four probable mechanisms of prodigiosin are proposed as pH modulators, cell cycle inhibitors, DNA cleavage specialists and regulators of mitogen activated protein kinase. These particles when combined with some other anticancer specialists can incredibly help in combatting malignant growth. Impacts of human carcinoma cells by the prodigiosin treatment were researched by Kavitha *et al.*, [6] and their significant outcomes were found. A variety of prodigiosin concentrations were examined against the percentage of malignant cells declination. Similarly, Prodigiosin was likewise demonstrated to be associated with apoptosis of haematopoietic disease cell [7]. *Spodoptera litura* is one of the harvest destructing insect which is usually controlled by a significant weapon derived from *Bacillus thuringiensis* toxin viz. Cry1C. Insecticidal activity of the toxin was found to be enhanced when cry1Ctoxin was combined with prodigiosin. Parani and Saha [8] optimized the production medium for production of higher concentration of prodigiosin which was incorporated with the casein-enrichment medium provided with 4% vegetative oil combination (sunflower, coconut and olive oil). Similarly, contagious action was likewise investigated against the fungus like *Helminthosporium sativum*, *Fusarium oxysporium* and *Rhizoctonia solani*.

The pigment of prodigiosin likewise isolated from *Streptomyces spectabilis* was confirmed to be antimalarial. The bacterium *Streptomyces spectabilis* was confirmed to be producing meta cyclo-prodigiosin which is 50% active against *Plasmodium falciparum* K1 without damaging normal cells [9]. These critical outcomes can help in destroying malarial parasites. Production of enzymes like lipase has also been observed in *S. marcescens*. The enzymes are demonstrated to be active at the optimal conditions at pH 8.0 and 45°C temperature. The molecular weight of the proteins was discovered to be 65 Da. with significant stability in many water miscible solvents [10].

Methicillin–resistant *Staphylococcus aureus* (MRSA) is a gram positive bacterium, a significant cause for medical infections worldwide and is responsible for large number of deaths per annum than HIV-AIDS in the US [11]. Furthermore MRSA is the main reason for blinding eye with the diseases including endophthalmitis and keratitis [12]. The *Serratiasp.* has been accounted for to produce antibioticsincluding a carbapenem, prodigiosin and serratamolide [13-15].The current work tried to explore the utilization of prodigiosin from *Serratia marcescens* strain segregated from farm soil and are tried to prove the antimicrobial effectivity of prodigiosin pigment to hinder the development of MRSA strains.

MATERIALS AND METHODS:

COLLECTION OF SAMPLE:

The damped soil sample was collected in a sterile container from the rhizosphere region surrounding the roots of the banana tree.

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ISOLATION AND SCREENING OF PRODIGIOSIN PRODUCING *SERRATIA MARCESCENS*:

About 1g of the soil sample was added to the test tube marked 1 provided with 10 ml of sterile water. Then the sample was serially diluted till 10^{-1} using the test tubes containing 9 ml of sterile water. From each and every serially diluted tube0.1 ml of diluted sample was taken and spread plated over the nutrient agar plate by spread plate technique. The plates were then incubated at 37° C for 2-3 days. After incubation, the colonies containing significant red colour over the plate were picked and further subcultured on the separate nutrient agar plates. The grown cultures were further stored in refrigerator for further studies.

IDENTIFICATION OF *SERRATIA MARCESCENS* BY BIOCHEMICAL CHARACTERISTICS:

Pure cultures of the bacterial isolates were selected and morphologically distinct colonies are characterised by biochemical characterization and are identified using Bergey's manual of systematic bacteriology.

Gram staining:

The glass slide was cleaned by using ethanol. One drop of water was placed in the slide, smeared using a single colony and heat fixed. The heat fixed slide was added with 2 drops of crystal violet and allowed to stand for 1 minute. Followed by washing with distilled water the slide was added with gram's iodine solution and allowed to stand for 1 minute, then washed with distilled water. The cells were decolorised using ethanol drop wise and washed with distilled water. The slide was then added with 2 drops of saffranin and washed with distilled water after 1minute. Finally, the slides were air dried and observed under microscope at 100X magnification using oil immersion.

Motility test:

A drop of liquid broth culture was placed in a clean coverslip the edges greased with vaselline. The coverslip containing culture was made to attach to the clean glass cavity slide and turned upside down. The hanging culture attached to the slide was observed under microscope using 40X magnification power.

Indole test:

Indole test was performed by inoculating the culture in the sulphide indole motility (SIM) medium. Two clean test tubes were added with 5ml of SIM medium and are autoclaved. A loopful of test organism was inoculated into the test tube after medium solidification by stab inoculation and the other tube was served as control. After incubation at 37°C for 24 hrs, 0.5 ml of Kovac's reagent was added and observed for the result.

Methyl red:

Methyl red test was performed by inoculating the culture in the medium containing MRVP broth. Two clean glass test tubes were added with 5ml of MRVP broth and are autoclaved. A loopful of test organism was inoculated onto the MRVP broth of a test tube and other served as control. After incubation at 37°c for 24 hrs the inoculated tubes were added with 2 drops of methyl red and observed for the result.

Voges-Proskauer:

Methyl red test was performed by inoculating the culture in the medium containing MRVP broth. Two clean glass test tubes were added with 5ml of MRVP broth and are autoclaved. A loopful of test organism was inoculated onto the MRVP broth of a test tube and other served as control. After incubation at 37°c for 24 hrs

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the inoculated tubes were added with 8 drops of Barritt's reagent (A and B) and observed for the result.

Citrate utilization:

Citrate utilization test was performed by inoculating the culture in the medium containing Simmon's citrate agar. Two clean glass test tubes were added with 7ml of Simmon's citrate agar and are autoclaved. The slant is prepared in a manner so as the butt and slant are equal in length. The tube containing Simmon's Citrate agar medium was inoculated with one loopful of test organism by stab inoculation and the tubes were incubated at 37°c for 24 hrs. The results were interpreted by observing the colour change after respective incubation.

Catalase test:

The ability of the test organism to produce the enzyme catalase is detected by mixing a loop full of colony with a drop of H_2O_2 (Hydrogen Peroxide) placed over a clean glass slide. The drop is noted for effervescence and the results are noted.

PRESUMPTIVE TEST FOR PRODUCTION OF PRODIGIOSIN PIGMENT BY *SERRATIA MARCESCENS* ISOLATES:

About 1 ml of the enriched isolates was inoculated into LB (Luria Berterni) broth and the flasks were incubated at 37°C for 4 days. The flasks were observed virtually for pigment production by colour change of the medium. About 10 ml of the culture broth was centrifuged at 10,000 rpm for 10 minutes using cooling centrifuge. The obtained supernatant was discarded and the resulting pellet was re-suspended with acetone. The mixture was incubated in the shaker incubator for 1 hour so as to extract the pigment from the cell. The suspension was again centrifuged at 10,000 rpm for 10 minutes and the debris collected at the bottom was removed and the supernatant containing pigment was taken in two separate test tubes. The content of the first tube was acidified with few drops of concentrated hydrochloric acid and the second tube was alkalinized with a drop of ammonium solution. Then the colour change was observed on both the tubes and the result were noted respectively [16].

EXTRACTION OF PRODIGIOSIN PIGMENT :

About 10 ml of the isolates inoculated in 250 ml of sterile LB broth and the flasks were incubated in shaker incubator at 27°C for 72 hrs. After incubation extraction of prodigiosin was initiated by centrifuging the broth at 10,000 rpm for 15 mins using cooling centrifuge. The obtained supernatant was discarded and the pellet containing the pigment was taken for further analysis. The pigment was extracted from the cell pellet by adding 4 volumes of acetone to the cell suspension and is incubated under shaking for 3 hrs at room temperature. Then the suspension was centrifuged again at 10,000 rpm for 10 min. The obtained sediment of cell debris was washed twice by re-suspending in 50 ml of acetone with continuous shaking for 30 min and is centrifuged again. The washings were finally combined with the supernatant from the original centrifugation. Pigments were extracted from small the supernatant by mixing thoroughly with 1 volume of acetone solution followed by 2 volume of petroleum ether using separatory funnel. The acetone with the water phase was removed and the prodigiosin which was separated in the petroleum ether phase was collected. The suspension was transferred to a sterile petridish and the petroleum ether was allowed to evaporate at 30°C to 40°C. The dry pigment obtained after evaporation was scrapped from the petridish and are used for further analysis [17].

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ESTIMATION OF THE PRODIGIOSIN PIGMENT:

The amount of prodigiosin produced was estimated by the absorption pattern of the suspension at different wavelengths 490nm and 610 nm. The results were studied after above mentioned time intervals. The unit of prodigiosin produced was estimated using the formula [18].

$$\text{Prodigiosin} = \frac{OD\ 490 - (1.381 * OD\ 610)] * 1000}{OD\ 610} \text{ unit/cell} \quad \text{(Eq.1)}$$

OD= Optical density; OD 490= pigment absorption; OD 610= bacterial absorbance; 1.381=constant.

OPTIMIZATION OF CULTIVATION CONDITIONS FOR ENHANCING PRODIGIOSIN PIGMENT:

Preparation of preculture broth:

Preculture of the *Serratia marcescens* isolates were prepared by inoculating one loopful of culture in20 ml of sterile peptone broth and are incubated at room temperature for overnight. The enriched culture was then used for optimization the cultivation conditions.

Effect of different media on prodigiosin production:

The prodigiosin production and growth characteristics of the test organism were monitored using different production media like Nutrient broth, LB broth, peptone glycerol broth. Different production media were prepared and autoclaved. Then 1ml of fresh preculture was inoculated into each sterilized media separately. The amount of prodigiosin pigment produced was estimated at time intervals (24 hrs, 48 hrs, 72 hrs and 96 hrs) [19].

Effect of incubation time on prodigiosin production:

Effect of incubation period on the growth and prodigiosin production are quantified by inoculating 1ml of enriched preculture to the sterile LB broth. The inoculated flaks were incubated at room temperature at different time intervals (24 hrs, 48 hrs, 72 hrs and 96 hrs). The amount of prodigiosin produced was monitored at therespective time intervals of incubation [20].

Effect of different carbon sources on prodigiosin production:

The effect of various carbon sources (glucose, sucrose, lactose and fructose) on the growth and prodigiosin production of test organism was monitored. About 1% w/v concentration of the different carbon sources were supplemented to the sterile LB broth separately. To the sugar supplemented medium 1ml of the enriched culture was inoculated and was incubated at room temperature. The amount of pigment produced was estimated periodically at time intervals (24 hrs, 48 hrs, 72 hrs and 96 hrs).

Effect of pH on prodigiosin production:

The pH condition suitable for the microbial growth and prodigiosin production of the test organism was determined. LB broth was prepared with different pH range (6, 7, 8 and 9) and autoclaved. To the sterile LB medium about 1ml of enriched preculture was inoculated and the flasks were incubated at room temperature. The amount of pigment produced was estimated at time intervals (24 hrs, 48 hrs, 72 hrs and 96 hrs).

Effect of temperature on prodigiosin production:

The effect of temperature on the growth and prodigiosin production are estimated by incubating the inoculated flask under various temperatures (28°C, 30°C, 35°C

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and 37°C). The LB broth was prepared in several flasks and is inoculated with 1 ml of enriched medium. The inoculated flasks were then incubated at different temperature and the amount of pigment produced was estimated at different time intervals of 24 hrs, 48 hrs, 72 hrs and 96hrs.

Effect of oil substrates on prodigiosin production:

The effect of different oil substrates to enhance pigment production was analysed by adding 2% of various oils (castor oil, sesame oil, coconut oil and neem oil) into the LB broth. The LB broth was prepared and added with various oils and are sterilized. Then the broth was inoculated with 1ml of the preculture and the flasks are incubated at room temperature. The pigment production was estimated at different time intervals of 24 hrs, 48 hrs, 72 hrs, and 96 hrs.

Effect of nitrogen sources on prodigiosin production:

The effect of various nitrogen sources (urea, peptone, beef extract and yeast extract) on the microbial growth and prodigiosin production by the test isolate are optimized. LB broth was prepared with 0.5%w/v of different nitrogen source in the separate flask and it are sterilized. Then the broth was inoculated with 1 ml of the enriched culture and incubated at room temperature. The amount of pigment produced was estimated at time intervals of 24 hrs, 48 hrs, 72 hrs and 96 hrs.

Effect of salt concentration on prodigiosin production:

The effects of various salt concentrations (0.5%, 1%, 1.5%, 2% of NaCl) on pigment production by test organism was evaluated and optimized. To the LB broths prepared various concentration of salt was added and sterilized. To the autoclaved LB broth 1ml of enriched culture was inoculated and incubated at room temperature. Then the amount of prodigiosin produced was estimated at different time intervals (24 hrs, 48hrs, 72hrs and 96 hrs).

Characterization of prodigiosin by FTIR (Fourier Transform Infrared Spectroscopy) analysis:

The purified prodigiosin was subjected to FTIR spectroscopic analysis (shimadzu), equipped with KBr beam splitter with the wavelength and resolution of 4000-350 $cm^{-1}$and 0.9 cm^{-1} resolution [21].

Analysis of antimicrobial activity of prodigiosin against Methicillin resistant *Staphylococcus aureus*:

Preparation of prodigiosin impregnated discs:

The prodigiosin impregnated discs were prepared by using sterile discs. The autoclaved discs were added with different concentration of prodigiosin pigment such as 50µl, 100µl and 150µl. Then the discs were dried at about 30°C for 20 mins in a hot air oven.

Antimicrobial activity of the produced prodigiosin

Mueller Hinton plates were prepared and autoclaved. The inoculum was prepared by inoculating a loopful of MRSA culture to 5 ml of sterilized peptone broth and are incubated for 15 mins. To the solidified Muller Hinton agar medium the enriched MRSA culture was swabbed and the prodigiosin impregnated discs prepared were placed onto it. The plates were incubated at 37°C for 24 hrs and the zone of inhibition formed after incubation was measured.

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Table 1. Morphology and biochemical characteristics of *Serratia marcescens*.

S.No.	Variables	Results
1.	Colony shape	Round
2.	Colony size	1 mm
3.	Colony colour	Red
4.	Cell motility	Motile
5.	Cell shape	Rods
6.	Grams staining	Gram Negative
7.	Indole test	Negative
8.	Methyl Red	Positive
9.	Voges Proskauer	Negative
10.	Citrate utilization test	Negative
11.	Catalase	Positive

Table 2. Estimation of Pigment production by absorption at 490 nm(units/ cells).

Hours of Incubation	Amount of Prodigiosin produced (mg/cells)		
	Nutrient broth	Peptone glycerol broth	LB broth
24 hrs	0.68	0.68	0.72
48 hrs	0.58	0.74	0.96
72 hrs	1.17	0.96	1.48
96 hrs	0.98	0.81	1.15

Table 3. Zone of inhibition (mm) of different concentration of prodigiosin against MRSA.

S.NO	Concentration of prodigiosin	Zone of inhibition(mm)
1.	50µl	1.5mm
2.	100µl	6mm
3.	150µl	8mm

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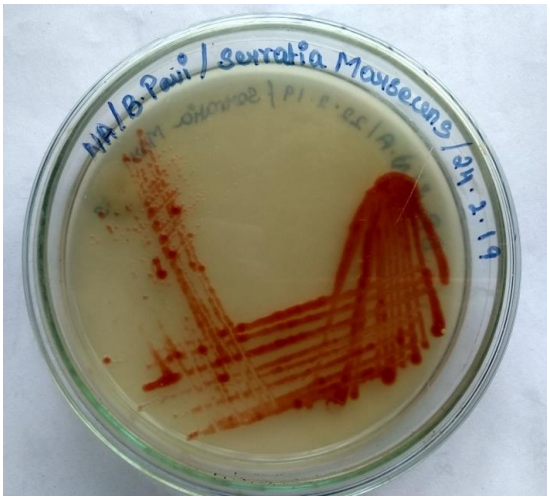

Plate 1 – Isolation of *Serratia marcescens* from soil sample

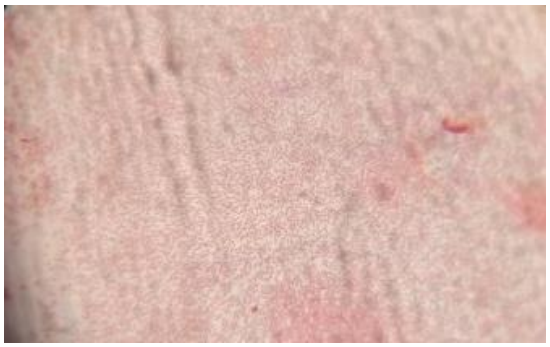

Plate 2 – Gram stained cells of *Serratia marcescens* under 100X magnification

(a) (b) (c)

Plate 3 - Biochemical identification of *Serratiamarcescens.* a) Indole test b) Citrate utilization) Methyl red and Voges proskauer.

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(a)

(b)

Plate 4 – Presumptive test for prodigiosin production a) Addition of HCl b) Addition of Ammonia.

(a)

(b)

(c)

Plate 5 – Optimization of prodigiosin production using different media a) Nutrient broth b)Peptone glycerol broth c)LB broth.

Plate 6- Inhibitory activity of prodigiosin against MRSA (Methicillin resistant *Staphylococcus aureus*)

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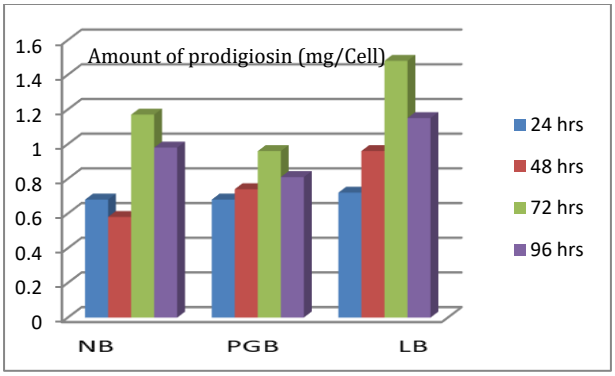

Fig 1.Estimation of prodigiosin pigment by absorption at 490nm.

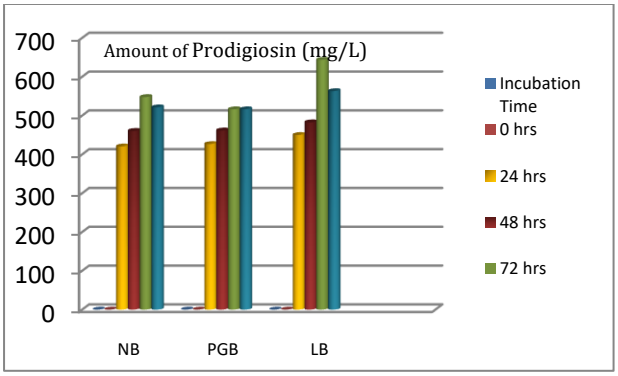

Fig 2: Effect of different media on prodigiosin production.

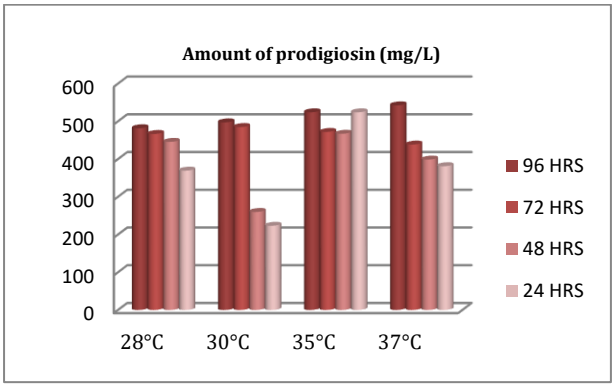

Fig 3. Effect of incubation time on prodigiosin production

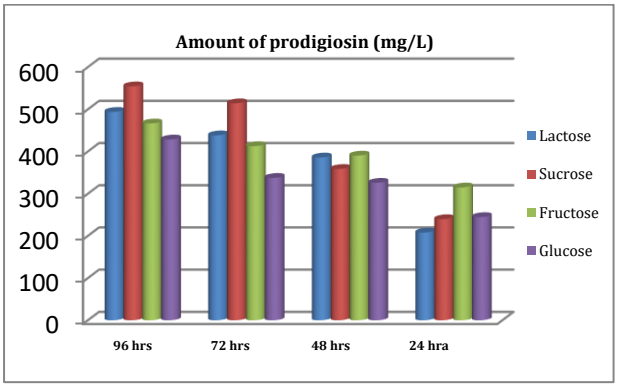

Fig 4. Effect of carbon sources on prodigiosin production

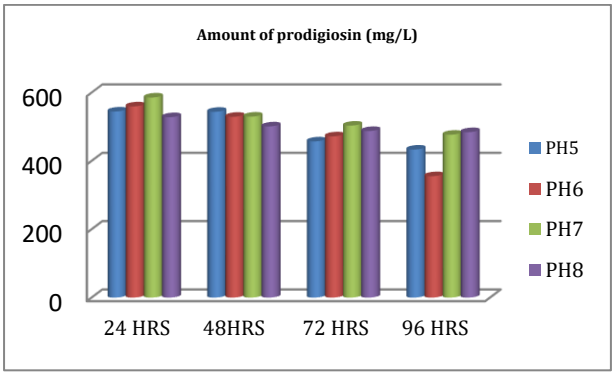

Fig 5. Effect of pH on prodigiosin production

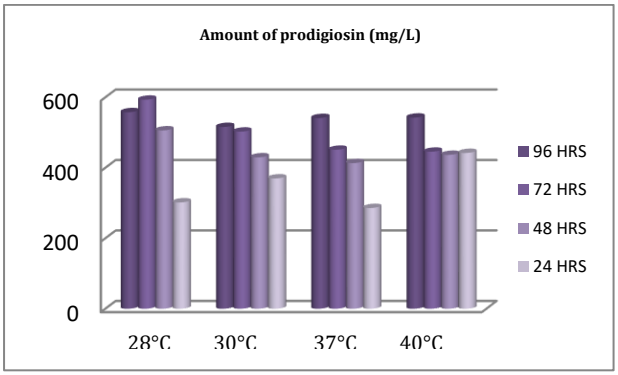

Fig 6. Effect of temperature on prodigiosin production.

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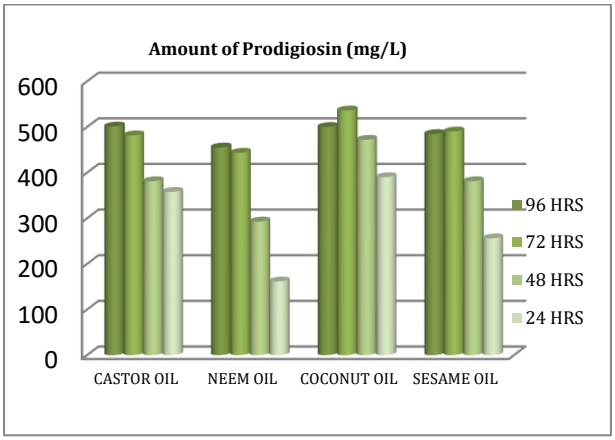

Fig 7. Effect of oil substrates on prodigiosin production

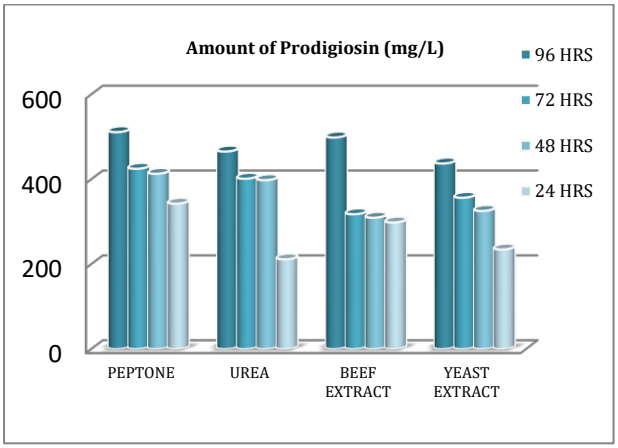

Fig 8.Effect of nitrogen sources on prodigiosin production.

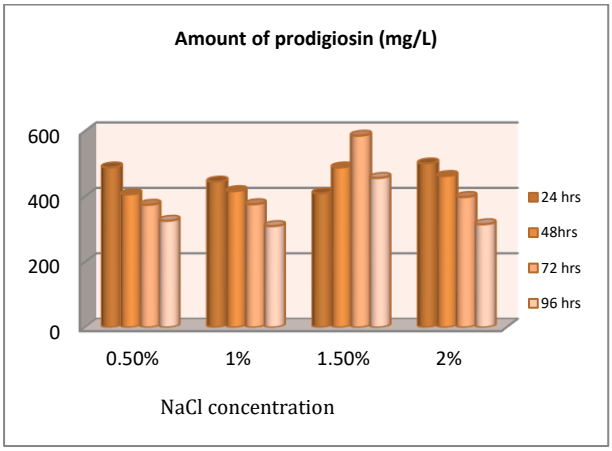

Fig 9.Effect of salt concentration on prodigiosin production.

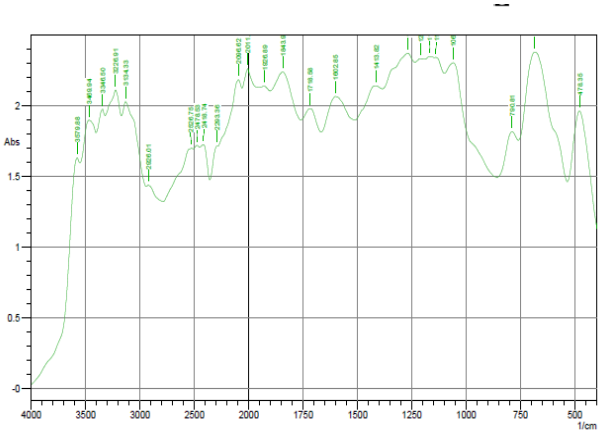

Fig 10.FTIR spectrum of prodigiosin pigment.

RESULTS AND DISCUSSION

ISOLATION AND SCREENING OF PRODIGIOSIN PRODUCING *SERRATIA MARCESCENS*

The microbes employed for the present investigation was isolated from the damped soil. A variety of microbes along with the pigmented strains are isolated with their ability to thrive under various environmental conditions. Distinct colonies *Serratia marcescens* with red pigmenting ability on nutrient agar at 28±2°C were isolated from the samples. The obtained results suggested that the damped soil especially from the rhizosphere region is the thriving source for the isolation of innumerable variety of pigmented bacteria with biomedical potentiality in moderate proportions. And the probable high variety of bacteria under high cell density along with *Serratia sp.* was due to the increase in human activity and visit in enormous number thus resulted in environmental pollution with bacterial resistance with substantial increase. Thus the potentiality

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of the isolates from the basic environmental sources confides on the biological products and metabolites with antimicrobial capability. And those potentially available compounds can also be applied towards many industries.

In the present work, the pigment producing bacterium *Serratia marcescens* was isolated from the soil sample and it was selected for further screening purposes. Production of prodigiosin pigment was confirmed by cultivating the isolates in nutrient agar plates. The culture which shows bright pigmentation in the agar was selected based on visual observation (Plate 1). The production of red pigment prodigiosin was selected based on the intensity of red colour produced in the liquid medium.

IDENTIFICATION OF *SERRATIA MARCESCENS* ISOLATES BY BIOCHEMICAL CHARACTERISTICS:

The isolates were identified by analysing the biochemical and morphological characteristics based on Bergey's Manual of Determinative Bacteriology [22]. The obtained morphological characteristics with the biochemical results of the organism it was confirmed that the organism was *S. marcescens* (Table 1& Plate 2, 3).

PRESUMPTIVE TEST FOR PRODUCTION OF PRODIGIOSIN PIGMENT BY *SERRATIA MARCESCENS* ISOLATES:

Presumptive test for prodigiosin was carried out by centrifuging the LB broth containing the culture. The cell pellet was then dissolved in acetone and centrifuged. The supernatant taken in the test tubes were acidified on the addition of Conc. HCl by forming deep pink color and were alkanized with ammonia solution by changing the colour to orange. Thus the compound extracted showed positive result on presumptive test for prodigiosin (Plate 4).

EXTRACTION OF PRODIGIOSIN PIGMENT:

The prodigiosin pigment was highly insoluble in water, thus the prodigiosin pigments are usually extracted by using solvents like acetone [23].The was acetone found to be best for the extraction which yield powdered pigment. The polarity of the solvents has to be considered for the extraction purposes. The polarity of the solvents could be increased by the addition of the solvents like ethyl acetate.

ESTIMATION OF PRODIGIOSIN PIGMENT:

The Prodigiosin pigment can be estimated by using the spectrophotometer between the range of 400–800 nm and the maximum peak was observed between the wave length of 490nm. The optical density of the pigment was estimated at various time intervals (24, 48, 72 and 96 hrs) and was quantified. The pigment produced by *S. marcescens* produce yellow-orange in alkaline solution with an absorption maximum at 470 nm [24].

As the incubation time increases the pH of the medium found to be decline and thus prolong incubation is not helpful in prodigiosin production. In the present study, the amount of prodigiosin produced in nutrient broth, peptone glycerol broth, LB broth by *Serratia marcescens* was estimated.

Along with the pigment growth of the bacteria was also monitored spectrophotometrically at regular intervals. The maximum amount of prodigiosin was produced by cultivating the *Serratia marcescens* in LB broth for 72hrs (1.48 mg/cell). The amount of pigment absorption was exhibited in table 2 and fig.1.

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OPTIMIZATION OF CULTIVATION CONDITIONS FOR ENHANCING PRODIGIOSIN PIGMENT:

Effect of different media on prodigiosin production:

The culture was inoculated at different media like nutrient broth, peptone glycerol broth and LB broth. Comparing the result LB broth will produce better prodigiosin pigment at 72 hrs (643.82mg/L). Hence LB broth will selected for further optimization studies. The estimation was given in bar diagram (Fig:2 &Plate 5).

Effect of incubation time on prodigiosin production:

The OD value of the culture was observed using calorimeter at different incubation period like 24 hrs, 48 hrs, 72 hrs and 96 hrs. The result showed maximum pigment production at the 96 hrs of incubation in 37°C (549.29 mg/L). The production was found to commence after 24 hours of incubation and its production increased with the increase in incubation period (Fig.3).

Effect of carbon sources on prodigiosin production:

LB broth containing glucose favoured highest production of prodigiosin pigment that increased every 24 hours of incubation. After 96 hours of incubation the concentration of prodigiosin was found to be (554.46 mg/l). The OD value was taken in calorimetrically followed by lactose, glucose, sucrose and fructose (Fig 4).

Serratia marcescens strains are demonstrated for their ability to utilize various carbon sources for its growth and prodigiosin production. As per the observation of Kurbanoglu *et al.* [25] as the glucose was supplied as a carbon source, *S. marcescens* MO-1 significantly decreased the production of prodigiosin. It has been demonstrated that the carbon sources like glucose and maltose exhibits supressive effect on the synthesis of prodigiosin due to catabolite repression and decrease in pH [26,27]. Since the *S. marcescens produce the alloenzyme* glucose-6-phosphate dehydrogenase which involves in inhibition of prodigiosin synthesis [26]. Utilization of glycerol and mannitol in the production medium was proved to enhance the yield of prodigiosin. While compared to glycerol mannitol was more suitable for the bacterial growth and prodigiosin production [28].

In the current study, it is evident that sucrose acts as a better source of substrate in enhancing pigment production in nutrient broth. Higher amount of prodigiosin (554.46 mg/L) was produced by *Serratia marcescens* strain when supplemented with sucrose in the nutrient medium. As per the observation of Sundaramoorthy *et al.* [29], the addition of sugar molecules like maltose, sucrose or lactose could enhance the pigment production and yield. But the addition of glucose to the medium was proved to be ineffective due to the activities of catabolite repression.

Effect of pH on prodigiosin production:

The parameter pH of cultivation medium is highly influential on the pigment production by microbes. Whereas, the colour intensity of the pigment could be altered based on the change in pH [30]. In the present work, the impact of pH on the prodigiosin production has been investigated using various pH values like 6, 7, 8 and 9.The maximum production of prodigiosin pigment was attained at the pH 7 with the yield of 585.55 mg/l. Lowest yield of prodigiosin pigment was produced at the conditions with pH 6, pH8, and pH9 (Fig 5).

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Effect of temperature on prodigiosin production:

The pigment biosynthesis is significantly affected by the impact of physiological parameter like temperature [31]. The constant incubation time of 72 hrs was used for the prodigiosin by strains of *Serratia marcescens* production at different temperature by 28°C, 30°C, 37°C and 40°C. The highest yield of prodigiosin pigment production was observed at 28°C (593.12 mg/l) at 72 hrs of incubation (Fig.6).

It was determined by Hejazi and Falkiner [31] that the pigment production was completely inhibited at the temperature of 45°C, whereas the pigment production was greatly decreased by increase in temperature. Thus, 30°C was confirmed to the optimum temperature for the prodigiosin pigment production. Eventhough, 35°C was very prominent in enhancing the cell density of the organism the pigment production is lower than that of temperature 30°C. Similarly, Khanafari *et al.* [32] and Giri *et al.* [33] also proved that optimum temperaturefor pigment production was 28°C and 30°C respectively.

Effect of oil substrates on prodigiosin production:

The production of prodigiosin by the *Serratia marcescens* is been impacted by the fatty acid containing substances like sesame seed and peanut seed oil [32]. These compounds are rich in the amount of saturated fatty acids which are greatly responsible for the production of prodigiosin pigment. The concentration of saturated fats in the pea nut oil is greater than that of the sesame oil, thus results in higher yield of the pigment with higher cell density of the *Serratia* strains [32].

In the present investigation various oil substrates are used. The OD value of the pigment produced by coconut oil substrate was higher with the yield of 536.05 mg/l. The maximum pigment production was attained at 72 hrs of incubation, which was then gradually decreased. The other oil substrates like neem oil, sesame oil, gingelly oil showed least amount of pigment production than the coconut oil (Fig. 7).

Effect of nitrogen sources on prodigiosin production:

Some physical factors are highly influential upon the synthesis of secondary metabolites like microorganisms. Among them nutritional parameter like nitrogen has dragged severe interest in designing low cost media in industry by the researchers. Framing of inexpensive media provided with great importance is needed for industries in economical perspective [34].

In the present work, different nitrogen sources like peptone, urea, yeast extract and beef extract was used for prodigiosin production by *Serratia marcescens*. Peptone supported thepigment production by producing maximum yield by 511.43 mg/l at 96 hrs of incubation. In contrary least prodigiosin production was observed in the medium with urea, which was followed by yeast extract and beef extract respectively (Fig.8).

Effect of salt concentration on prodigiosin production:

Different concentration of NaCl salt like 0.5%, 1%, 1.5%, 2% was used for pigment production. The maximum pigment production was observed in 1.5% salt concentration (585.81mg/l) at 72 hrs of incubation (Fig.9).

CHARACTERISATION OF PRODIGIOSIN BY FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR):

The absorption band observed at the wave number of 3289 Cm^{-1} represents the

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stretching vibrations of N-H aromatic ring, band at 2852 Cm^{-1} representsthe C-H stretching vibrations of aliphatic, and the band at wave number 1650 Cm^{-1} signifies the presence of N-H stretching aromatic vibrations [35]. Similarly, while analysing the FTIR peaks of the obtained red pigment, absorption peaks signifying strong bands were observed at the 3232.91Cm^{-1}, 2926Cm^{-1}, 1602.65 Cm^{-1}. Thus, as per the results obtained the prodigiosin compound signifies the stretching vibrations of the N-H aromatic ring and C-H aliphatic compounds. These results indicated that the produced pigment's pattern is similar to that of prodigiosin. The absorption spectrum representing different range of absorption peaks were depicted in the figure 10.

ANALYSIS OF ANTIMICROBIAL ACTIVITY OF PRODIGIOSIN AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*:

Eventhough the prodigiosin pigment did not encountered defined physiological role, it has been confirmed to have antimicrobial properties especially against Gram positive organisms [36]. The present study affirmed that the secondary metabolites produced by *S.marcescens* showed efficient antimicrobial property against the MRSA isolates. The extracted pigment (150 µl) showed higher inhibitory action (10mm) towards MRSA. The picture illustrating zone of inhibition with distinct clearance has been depicted in the Plate 6. The zone was measured from the edge of the MRSA strains. The zone of inhibition at different concentration for prodigiosin pigment was given in table 3.

CONCLUSION

The process of optimizing the growth conditions of *Serratia marcescens* for prodigiosin production exhibited significant effect while provided with sucrose as carbon source. Addition of oil was found to be very promising in the production of prodigiosin. The crucial parameter in prodigiosin production is the ratio of carbon and nitrogen in the growth medium. Studies using different nitrogen sources in the growth medium contribute greater prodigiosin accumulation with confluent bacterial growth. Higher inhibitory action towards MRSA strain with distinct clear zone of inhibition proved the clinical efficacy of the prodigiosin produced

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